Published June 1, 1989

Interactions of Misfolded Influenza Virus Hemagglutinin with Binding Protein (BiP)

Stella M. Hurtley, David G. Bole, Helana Hoover-Litty, Ari Helenius, and Constance S. Copeland

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. We have characterized the association between the binding protein, BiP (also known as GRP 78), and misfolded forms of the influenza virus hemagglutinin precursor, HA0. BiP is a heat-shock–related protein that binds to unassembled immunoglobulin heavy chain and to a variety of misfolded proteins in the lumen of the ER. A small fraction (5–10%) of newly synthesized HA0 in CV-1 cells was found to be misfolded and retained in the ER. When glycosylation was blocked with tunicamycin, all of the HA0 produced was similarly misfolded. The misfolded HA0 was retained as relatively small (9–25-S) complexes associated with BiP. In these complexes the top domains of HA0 were correctly folded judging by their reactivity with monoclonal antibodies, but the polypeptides were cross-linked via anomalous interchain disulfides. The association with BiP was noncovalent and easily broken by warming to 37°C or by adding ATP to the lysate. Pulse–chase experiments showed that HA0's self-association into complexes occurred immediately after synthesis and was followed rapidly by BiP association. The misfolded, BiP-associated HA0 was not transported to the plasma membrane but persisted as complexes in the ER for a long period of time before degradation ($t_{1/2}$ = 6 h). The results suggested that BiP may be part of a quality control system in the ER and that one of its functions is to detect and retain misfolded proteins.

Most secretory and membrane glycoproteins of eukaryotic cells are cotranslationally translocated into the lumen of the ER from which they proceed to the Golgi complex and other destinations on the secretory pathway. If, for some reason, they fail to obtain the correct tertiary or quaternary structure they are usually retained in the ER and eventually degraded. The "quality control" mechanisms involved in recognizing, retaining, and degrading misfolded and misassembled proteins are not understood, but several studies have suggested that a heat-shock–related protein, BiP (or GRP 78), may be involved (Bole et al., 1986; Dorner et al., 1987; Kassenbrock et al., 1988; Kozutsumi et al., 1988). BiP, a 78-kD soluble nonglycosylated resident protein of the ER, has also been proposed to serve as a folding and assembly factor for oligomeric proteins (Gething et al., 1986; Munro and Pelham, 1986; Pelham, 1986, 1988*b*).

To follow the fate of misfolded plasma membrane glycoproteins and characterize their interactions with BiP, we have studied a viral spike glycoprotein, the influenza hemagglutinin precursor, HA0. The structure of the hemagglutinin trimer is known at a 3Å resolution (Wilson et al., 1981); it consists of a globular head domain, composed of HA1, attached to a stem domain, consisting mostly of HA2. HA0 is an 84-kD transmembrane glycoprotein synthesized in the ER of infected or transfected cells. Newly synthesized HA0 monomers undergo rapid initial folding, trimerize with a $t_{1/2}$ of 7–10 min and are then transported to the Golgi complex ($t_{1/2}$ of 10–20 min) (Copeland et al., 1986, 1988; Gething et al., 1986; Yewdell et al., 1988). We have previously shown that a small fraction (5–10%) of HA0 synthesized in CV-1 cells does not reach the Golgi complex, judging by the lack of processing of its N-linked carbohydrate chains. This HA0 population reacts with a monoclonal antibody (A1) directed to an epitope that is not exposed in monomers or mature trimeric forms of HA0. Immunofluorescence has shown that the A1-reactive HA0 is localized in the ER. We have concluded from these results that a fraction of HA0 apparently misfolds during or after synthesis, and, being misfolded, is retained in the ER (Copeland et al., 1986). Our immunoprecipitation experiments, as well as those of Gething et al. (1986) using HA0 of a different strain, showed, moreover, that BiP was associated with at least some of the misfolded HA0 chains.

In this paper we have further characterized both the spontaneously misfolded HA0 as well as aberrant HA0 molecules synthesized in the presence of tunicamycin, a drug that inhibits N-linked glycosylation (Elbein, 1983). In both cases the folding defects appeared to be expressed mainly in the stem region of HA0 molecules. They resulted in rapid self-aggregation of newly synthesized HA0 molecules by interchain disulfide bonds and in noncovalent, permanent association with BiP. The complexes were retained in the ER and slowly degraded.

C. S. Copeland's current address is Department of Biology, Kline Biology Tower, Yale University, P. O. Box 6666, New Haven, CT 06511.

Published June 1, 1989

Materials and Methods

Cell Lines, Virus, and Antibodies

CV-1 monkey kidney cells expressing X:31 HA0 from the SV-40 late replacement vector SVEXHA (Doyle et al., 1986) were used 50 h after infection. Chinese hamster ovary (CHO) 15B cells were cultured and infected with influenza virus (X:31 strain) as previously described (Copeland et al., 1988).

The antihemagglutinin antibodies used have all been previously described. Monoclonal antibodies N1 and N2 are directed to trimer-specific epitopes at the tips of the HA0 molecule (Copeland et al., 1986). The rabbit polyclonal antiserum reacts with HA1 and HA2 polypeptides of mature or immature trimeric, monomeric, and misfolded forms of hemagglutinin, and with SDS-denatured or acid-treated hemagglutinin (Copeland et al., 1986). Antibody A1, which is directed to an epitope in the HA2 subunit, does not react with mature trimeric HA or HA0, nor does it react with the monomeric precursors to trimeric HA0. A1 does, however, recognize SDS-denatured hemagglutinin and hemagglutinin which has undergone a characteristic low pH–induced conformational change involved in its membrane fusion activity (Copeland et al., 1986; Boulay et al., 1988). Thus the A1 epitope is normally masked, but becomes exposed upon specific or nonspecific unfolding reactions. The anti-BiP monoclonal antibody was previously characterized by Bole et al. (1986).

Antibodies 11/4 (Webster and Laver, 1980), HC159X, HC3, HC19, HC68X, HC3i, HC125X, and HC100 have been previously described (Daniels et al., 1984, 1987). 11/4, HC159X, and HC3 recognize the "A" epitope; HC19, HC110X, HC68X, HC3i, and HC125X recognize the "B" epitope; HC100 recognizes the "E" epitope (Wiley and Skehel, 1987).

All immunoprecipitations were carried out according to Webster et al. (1983). To stabilize the HA0–BiP interaction, immunoprecipitation and all the washes were, however, performed at 4°C.

Metabolic Labeling and Cell Lysis Conditions

Radioactive labeling and lysis were as described (Copeland et al., 1986, 1988) with the following additions. For tunicamycin treatments, tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) was added to medium at 5 μg/ml final concentration from 5 mg/ml stock in DMSO. Control cells received an equal amount of DMSO. Pretreatments with tunicamycin were carried out for 20–60 min before metabolic labeling. To inhibit oxidation of free sulfhydryl groups, 50 mM iodoacetamide or 20 mM *N*-ethylmaleimide was frequently included in the lysis buffer. ATP was eliminated from lysates by the addition of 10 U/ml hexokinase and 0.2 mM D-glucose, and proteolysis was inhibited by inclusion in the lysis buffer of 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin, from a stock of 10 mg/ml of each in DMSO.

EM Immunocytochemistry

EM immunocytochemistry was by a modification of the methods of Brown and Farquhar (1984). Briefly, CHO 15B cells were infected with X:31 influenza virus, treated with tunicamycin or DMSO from 4 to 6 h after infection, and brought into suspension with trypsin. Culture medium was diluted 1:1 with fixative to give a final concentration of 50% alpha MEM, 4% FCS, 10 mM $NaIO_4$, 37.5 mM lysine, 25 mM $NaPO_4$, 2% formaldehyde, pH 6.3. Washed cells were adsorbed onto poly-L-lysine–coated microscope slides, permeabilized with 0.2% saponin, and stained with antibody A1 at 20 μg/ml followed by sheep anti–mouse horseradish peroxidase (Biosys) diluted 1:70. Postfixation and embedding were as previously described (Copeland et al., 1986).

Other Techniques

Sedimentation experiments were modified from the previously described protocols (Copeland et al., 1986) as detailed in the figure legends.

To check that the antibodies used were conformation specific, lysates were treated with 1% SDS and 5 mM DTT and incubated at 95°C for 5 min. Then they were cooled, and 4% Triton X-100 and 10 mM iodoacetamide were added; then immunoprecipitations were performed as usual.

When required, gel bands were quantitated using a digital gel scanner (Visage 2000). Control gels with increasing amounts of identical samples were used to test the linearity of the scanner response, and gel exposures within this range were scanned. In most cases, longer exposures were used for photography than for quantitation to allow for reproduction of faint bands.

Results

In most of our experiments, CV-1 cells expressing HA0 (X:31 strain) from an SV-40 late replacement vector were used. The cells were labeled with [^{35}S]methionine, lysed using Triton X-100, and subjected to immunoprecipitation. Immunoprecipitated, radiolabeled proteins were visualized after SDS-PAGE by fluorography. The antibodies used were (*a*) a polyclonal antiserum to influenza virus that reacts with all conformational forms of HA0; (*b*) monoclonal antibody A1, which recognizes an epitope in the stem domain of misfolded and acid-treated HA0; (*c*) monoclonals N1 and N2, which specifically bind to the tip epitope (site B) in correctly assembled, trimeric HA0; (*d*) a panel of monoclonals to the epitopes in the top domain of intact HA0; and (*e*) a monoclonal antibody that binds to and immunoprecipitates BiP from a variety of species. The association of BiP and HA0 was maintained by reducing ATP in the lysates by addition of hexokinase and glucose and by performing the immunoprecipitations at 4°C using mild washes. Because of these mild conditions, several background bands are evident in the precipitations.

It has been suggested that a fraction of influenza HA0 interacts with BiP in the ER of the infected or transfected cell (Copeland et al., 1986; Gething et al., 1986). To examine this interaction further and to try to assess its role in HA0 folding or transport, we characterized the association between BiP and a subpopulation of HA0 which reacts with the monoclonal antibody, A1. Fig. 1 shows immunoprecipitates from HA0-expressing cells which had been labeled for 60 min with [^{35}S]methionine in the presence or absence of tunicamycin. In Fig. 1 *A* the precipitated proteins were reduced before electrophoresis; in Fig. 1 *B* they were not. As previously reported (Copeland et al., 1986), the amount of A1-precipitable HA0 was ~5–10% of the HA0 precipitated by the polyclonal antihemagglutinin antibody (cf. Fig. 1 *A*, lanes *1* and *2*). Coprecipitating with the A1-HA0 was a slightly faster migrating protein that was only barely separated from the HA0 band (Fig. 1 *A*, lane *2*). It migrated in the position of BiP (Fig. 1 *A*, lane *3*). When anti-BiP antibodies were used some HA0 coprecipitated, confirming a molecular association between BiP and HA0. The amount of coprecipitation was variable but can be seen in Fig. 3 *B*, lane *Lysate a*. Although the coprecipitation of BiP and a subfraction of X:31 HA0 was quite reproducible (see Figs. 1, 3, and 4 and Copeland et al., 1986), quantitative interpretation of the results was hampered by the similar mobility of the HA0 and BiP bands.

Nonglycosylated HA0 Misfolds Quantitatively and Binds BiP

Since the amount of spontaneously misfolded HA0 was low and somewhat variable, we tested the effect of tunicamycin, which is known to cause misfolding of many glycoproteins by blocking N-linked glycosylation (Schlesinger and Schlesinger, 1987). We found that folding of the X:31 HA0 was disturbed in the absence of carbohydrate addition. In

1. *Abbreviation used in this paper:* CHO, Chinese hamster ovary.

Published June 1, 1989

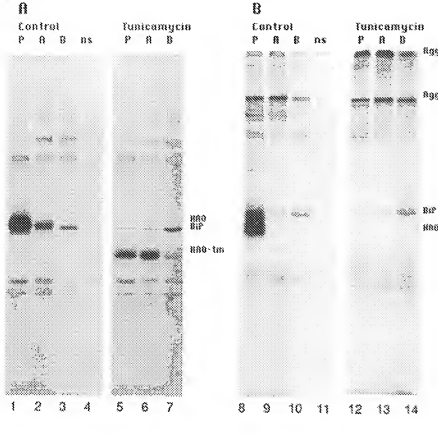


Figure 1. The misfolded HA0 complex. CV-1 cells expressing HA0 in the presence or absence of tunicamycin were labeled with [^{35}S]methionine for 1 h. Cell lysates were immunoprecipitated with a polyclonal antibody to HA (*P*), antibody A1 (*A*), anti-BiP (*B*), or with a nonspecific control antibody (*ns*). In *A* samples were reduced before SDS-PAGE; in *B* they were not reduced. HA0-tm is the nonglycosylated form of HA0. A number of background bands are apparent because of the low stringency washes used to preserve the BiP–HA0 complexes.

contrast to control cells, all of the HA0 synthesized was precipitable with antibody A1 (cf. Fig. 1 *A*, lanes *5* and *6*). BiP and HA0 bands were now well resolved by SDS-PAGE because of the faster migration of nonglycosylated HA0. The amount of labeled BiP coprecipitating with antibody A1 was increased, and up to 50% of the HA0 in the lysates could be precipitated with anti-BiP (Figs. 1 *A*, 3 *D*, and 4 *B*). Except for the lack of carbohydrate side chains, the misfolded HA0 molecules expressed in tunicamycin-treated cells were similar in their properties to those spontaneously formed in the absence of the drug: they were trypsin sensitive, did not react with trimer-specific antibodies N1 or N2, and were not transported to the plasma membrane (not shown).

To examine the intracellular localization of nonglycosylated HA0, we performed ultrastructural immunocytochemistry (Brown and Farquhar, 1984). Instead of CV-1 cells, we used influenza-infected CHO cells because they had superior morphology and the amount of misfolded HA0 in tunicamycin-free controls was low. In the tunicamycin-treated CHO cells the A1-reactive HA0 was detected in the ER and the nuclear envelope (Fig. 2 *A*). In addition, large vacuoles (labeled *L*) corresponding, presumably, to lysosomes or endosomes were stained. They were also stained in tunicamycin-free controls (Fig. 2 *B*) and probably represented endocytosed virions whose hemagglutinin had undergone the conformational change induced by the low pH in endocytic compartments (Doms et al., 1985; Kielian et al., 1986). No reaction product was observed in any part of the Golgi complex or on the plasma membrane (Fig. 2 *A*). Like the spontaneously misfolded HA0 (Copeland et al., 1986), the nonglycosylated HA0 was thus localized in the ER.

Structural Properties of Misfolded HA0

To evaluate the extent of molecular misfolding in the nonglycosylated HA0, we used 11 conformation-specific monoclonal antibodies to known epitopes on hemagglutinin (Daniels et al., 1984, 1987; Webster and Laver, 1980; Copeland et al., 1986). X-ray crystallography has shown that the mature trimer has three globular head domains comprised exclusively of HA1 subunits. These are located on top of a stem which is comprised mainly of HA2 subunits (reviewed by Wiley and Skehel, 1987). The major antigenic sites in hemagglutinin have been mapped to the globular head domains. We used antibodies that recognized three of these sites: A (loop, residues 142–146 in HA1); B (tip, residues 156–200 in HA1); and E (residues 77–85 in HA1) (see Wiley and Skehel, 1987). The antibodies to site B were found to fall into two groups: (*a*) N1, N2, and H125 were only able to precipitate trimers of HA and HA0; and (*b*) HC19, HC68, and HC31 precipitated both trimers and monomers. These groups have been previously defined by failure to react with variant HAs with single amino acid substitutions. Trimer-specific epitopes include residues 188–200 of HA1 which are located close to the subunit interface, whereas the other site B antibodies bind residues 156–159 of HA1 which are more distant from the interface.

The results summarized in Table I showed that all of the monoclonals precipitated nonglycosylated HA0 with the exception of the trimer-specific monoclonal antibodies directed to site B. None of the antibodies used in Table I recognized SDS-denatured HA0, indicating that they were all conformation specific. Thus the results suggest that the top

Published June 1, 1989

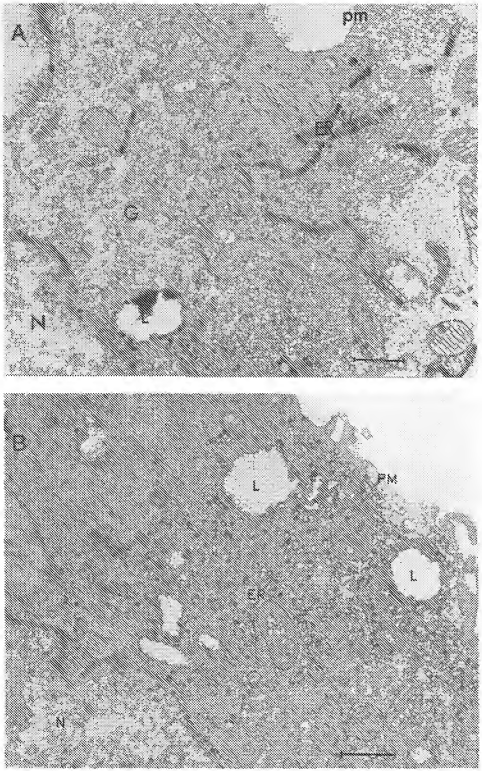


Figure 2. Immunocytochemical localization of misfolded HA0 tunicamycin-treated cells. CHO-15B cells were treated (*A*) or mock-treated (*B*) with tunicamycin for 2 h starting at 4 h after infection with influenza virus (X:31). Immunocytochemistry with antibody A1 was performed using the immunoperoxidase technique. In the presence of tunicamycin, the ER, nuclear envelope, and swollen vacuoles (probably lysosomes) were labeled. No label was seen in Golgi stacks or at the plasma membrane. *N*, nucleus; *G*, Golgi region; *pm*, plasma membrane; *L*, lysosome; and *ER*, endoplasmic reticulum. Bar, 0.6 μm.

domain of nonglycosylated HA0 was relatively correctly folded but had not trimerized. Given that the top domain appeared close to normal, the folding defect was likely to be in the stem domain. This was consistent with the reactivity with antibody A1 which binds to an epitope in HA2 of acid-treated and misfolded hemagglutinin (Copeland et al., 1986).

Misfolded HA0 Is Multimeric

The ectodomain of the correctly folded HA0 contains twelve cysteines all of which participate in intrachain disulfide bonds. The membrane-spanning and cytoplasmic domains contain five, presumably unbonded, cysteine molecules (Verhoeyen et al., 1980; Wilson et al., 1981). To investigate whether the folding defect resulted in the formation of aber-

*Table I. Immunoprecipitation of Misfolded and Correctly Folded HA0 with Conformation-specific Monoclonal Antibodies**

		Reactivity		
	Ref‡	Control HA0	Nonglycosylated HA0	Denatured HA0§
Trimer specific				
N2	1	+++	–	–
N1	1	+++	–	–
HC125	3	++	–	–
Site A				
11/4	4	+++	+++	–
HC159	3	+++	+++	–
HC3	3	+++	+++	–
Site B				
HC19	3	+++	+++	–
HC68	3	++	+	–
HC31	3	++	++	–
Site E				
HC100	2	+++	+++	–
Misfolded/acid-treated				
A1	1	–	+++	–

* CV-1 cells expressing HA0 were labeled with [^{35}S]methionine for 1 h in the presence or absence of tunicamycin. Immunoprecipitations were performed as described in Materials and Methods.

‡ Reference in which the antibody was described: (1) Copeland et al., 1986; (2) Daniels et al., 1984; (3) Daniels et al., 1987; and (4) Webster and Laver, 1980.

§ HA0 in cell lysates was denatured before immunoprecipitation as described in Materials and Methods.

rant disulfide bonds, aliquots of the immunoprecipitates subjected to SDS-PAGE after reduction in Fig. 1 *A* were electrophoresed without prior reduction (Fig. 1 *B*). Whereas the BiP band migrated close to its normal position in the nonreduced gels (Fig. 1 *B*, lanes *9*, *10*, and *12–14*), no monomeric HA0 band was present (Fig. 1 *B*, lanes *9*, *12*, and *13*). Instead smears were observed at the top of the stacking and separating gels (Fig. 1 *B*, *Aggregate*). When eluted, reduced, and reelectrophoresed this large molecular weight material was found to consist only of HA0 (data not shown). To ascertain that the cross-linking did not occur during or after lysis, 20 mM *N*-ethylmaleimide or 50 mM iodoacetamide was added to the lysis buffer, without affecting the outcome of the experiment. In addition, the same result was obtained if cells were treated with 50 mM iodoacetamide before lysis. These experiments demonstrated that the misfolded HA0 molecules were cross-linked by interchain disulfides and that BiP was noncovalently associated with the complexes.

To estimate the size of the complexes, lysates from tunicamycin-treated and untreated cells were subjected to velocity gradient centrifugation in the presence of Triton X-100. Aliquots from gradient fractions were immunoprecipitated with antibody A1 (Fig. 3, *A* and *C*) and anti-BiP (Fig. 3, *B* and *D*). Misfolded HA0–whether synthesized in the presence or absence of tunicamycin–sedimented in a broad zone at 9–25 S (Fig. 3, *A* and *C*). In contrast, correctly folded HA0 trimers sediment at 9 S and monomers at 4.5 S (Doms and Helenius, 1986). The majority of labeled BiP sedimented at 4–5 S (Fig. 3, *B* and *D*, and data not shown) suggesting that it was a monomer. Some coprecipitating BiP was clearly present with misfolded HA0 in the heavier fractions of the gradients containing misfolded HA0, and most of it coprecipitated with antibody A1 (cf. Fig. 3, *C* and *D*, lanes *3* and *4*).

In separate experiments, we established that BiP association with misfolded HA0 was quite sensitive to experimental conditions. For example, if gradient centrifugation was performed at 20 instead of 4°C, or if cell lysates were incubated overnight at 20°C before immunoprecipitation, no association between misfolded HA0 and BiP could be detected. After BiP dissociation, misfolded HA0 sedimented essentially as before, suggesting that BiP did not constitute a major fraction of the complex's mass.

Taken together, the sedimentation results showed that the misfolded HA0 was present in a multimolecular complex where it was the major structural component. The sedimentation coefficient of 9–25 S corresponds to globular proteins with molecular masses in the 200–1,000-kD range. The average complexes could thus contain five or six HA0 monomers and one BiP. The detailed composition of the complexes remains, however, unclear, due to the instability of HA0–BiP association, the size heterogeneity, and the possibility that other unlabeled components were present.

Munro and Pelham (1986) have reported that the interaction of BiP with immunoglobulin heavy chain is dissociated by ATP and suggested that ATP and ADP modulate the attachment of BiP to various substrates. Addition of hexokinase and glucose to the lysates as an ATP-depletion system did not increase the amount of stable BiP–HA0 complexes observed. However, the addition of ATP to cell lysates at 4°C efficiently dissociated BiP from the HA0 complexes (Fig. 4, *A* and *B*). Other nucleoside triphosphates (GTP, CTP, and UTP) could not substitute for ATP, nor did ADP or AMP-PNP, a nonhydrolysable ATP analogue, have any effect. Fig. 4 *C* demonstrates the thermolability of the BiP–HA0 interaction in cell lysates: 5 min at 37°C causes the dissociation of BiP, even in the absence of ATP.

Kinetics of Formation and Degradation of Misfolded HA0

Finally, we determined in tunicamycin-treated cells how rapidly the misfolded HA0 was sequestered into aggregates, how soon it associated with BiP, and how long the complexes remained in the ER before degradation. Quantitation of the gels produced in a pulse–chase experiment indicated that cross-linking via disulfides of nonglycosylated HA0 occurred immediately after synthesis (Fig. 5, *upper panel*). It was not possible to reliably quantitate the smears at the top of the resolving and stacking gels; therefore we measured in the polyclonal immunoprecipitates the small amount of nonaggregated HA0 running into the nonreducing gel. None of the A1-precipitable HA0 entered the nonreducing gel. At the end of a 2-min pulse, <10% of total HA0 produced in the tunicamycin-treated cells was observed migrating as monomers in nonreducing gels, indicating that >90% had already aggregated. The formation of the A1 epitope and the binding to BiP (as determined by coprecipitation with anti-BiP) occurred somewhat later with $t_{\frac{1}{2}}$ = 3–4 min (Fig. 5, *upper panel*). Spontaneously misfolded HA0 behaved simi-

Published June 1, 1989

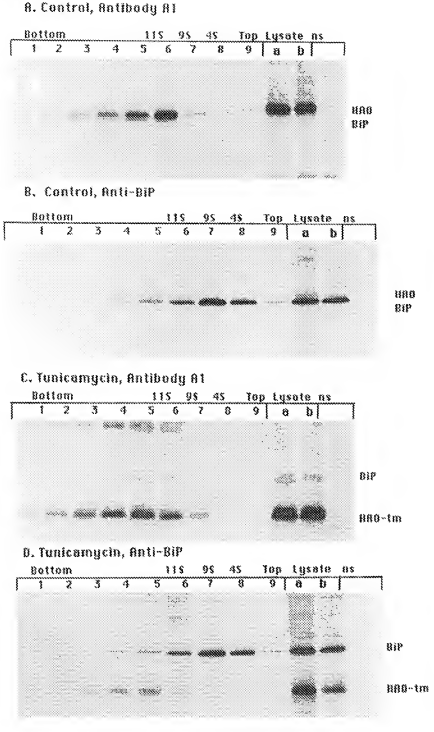


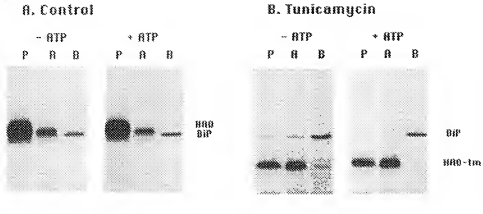
Figure 3. Sedimentation properties of misfolded HA0 and BiP. CV-1 cells expressing HA0 were labeled as in Fig. 1. Aliquots of the cell lysates were loaded onto a 5–25% sucrose density gradient containing 0.1% Triton X-100 with a 60% sucrose cushion and centrifuged at 100,000 *g* for 16 h at 4°C. Gradient fractions (lanes *1–9*) were immunoprecipitated with antibody A1 (*A* and *C*) or anti-BiP (*B* and *D*) and subjected to SDS-PAGE. Aliquots of the same lysates were also precipitated directly (lanes *a*) or after overnight incubation at 4°C to approximate the centrifugation conditions (lanes *b*). The sedimentation markers BSA (4*S*), trimeric HA0 (9*S*), and catalase (11*S*) were run in identical gradients; the bottom of the gradient corresponds to ~30 S. *HA0-tm*, nonglycosylated HA0; *ns*, immunoprecipitation with nonspecific control antibody.

larly; maximal BiP association and A1-epitope expression were observed within the first 2 min of chase (Fig. 5, *lower panel*). Misfolded HA0 thus separated from the normal pathway of HA0 transport and folding almost immediately after synthesis.

In both tunicamycin-treated and untreated cells, the relative intensities of coprecipitating HA0 and BiP bands remained constant during chase, indicating that the complexes were permanent (Fig. 5 and data not shown). The same result was observed when pulse-labeled tunicamycin-free cells were precipitated with polyclonal antihemagglutinin, which is capable of recognizing HA0 monomers. We have not observed transient BiP attachment to any form of HA0, whether precipitating with anti-BiP (Fig. 5) or polyclonal antihemagglutinin, even using pulses as short as 1 min.

To estimate the turnover rate of misfolded HA0 complexes, we followed the loss of BiP and misfolded HA0 with time. Tunicamycin-treated and untreated CV-1 cells at early times of infection were pulse-labeled for 20 min and chased for various times up to 8 h (Fig. 6). The time course of degrada-

Published June 1, 1989



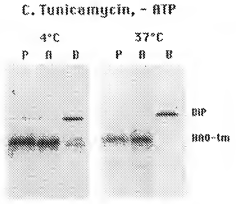


Figure 4. Effect of ATP on the BiP–HA0 association. CV-1 cells expressing HA were labeled as in Fig. 1. Cells were lysed in lysis buffers containing either 1 mM ATP or hexokinase/glucose (10 U/ml, hexokinase, 0.2 mM glucose). In *C* cell lysates containing hexokinase/glucose were incubated before immunoprecipitation for 5 min at 4 or 37°C. Immunoprecipitation was then performed using the polyclonal (*P*), antibody A1 (*A*), or anti-BiP (*B*) as indicated.

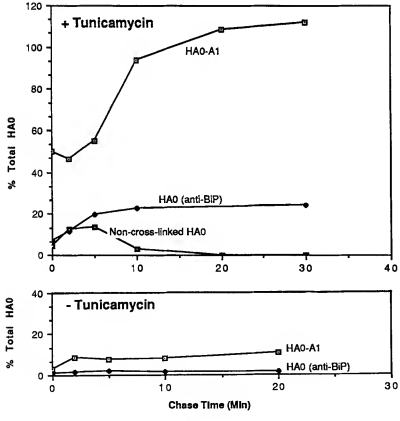


Figure 5. Rate of misfolding and complex formation. CV-1 cells expressing HA in the presence or absence of tunicamycin were pulse labeled with [^{35}S]methionine for 2 min and chased in the presence of excess cold methionine for various times up to 30 min. Cell lysates were immunoprecipitated with the polyclonal antibody (*total HA0*), antibody A1 (*HA0-A1*), or anti-BiP. The labeled HA0 precipitated was quantitated after SDS-PAGE by densitometry. The amount of non–cross-linked HA0 in the tunicamycin-treated cells was estimated by densitometry of HA0 monomer in nonreduced samples.

Published June 1, 1989

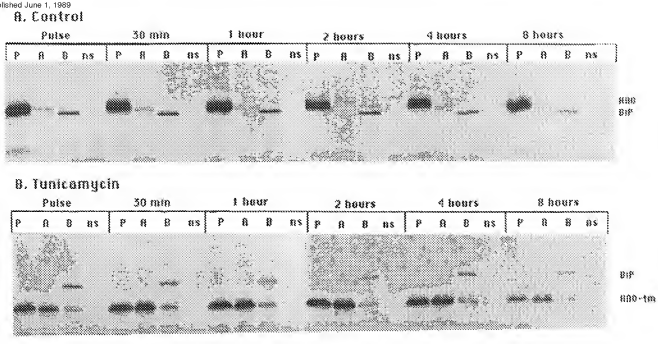


Figure 6. Lifetime of misfolded HA0. CV-1 cells expressing HA0 in the presence or absence of tunicamycin were pulse labeled with [^{35}S]methionine for 20 min and chased in the presence of excess cold methionine for various times up to 8 h. Cell lysates were immunoprecipitated with the polyclonal antibody (*P*), antibody A1 (*A*), anti-BiP (*B*), or nonspecific control (*ns*) as indicated. The gel exposures shown were chosen so that the HA0 bands were not over exposed. As a consequence, the BiP which coprecipitated with A1 is difficult to see.

tion was composed of a long lag phase, followed by a more rapid degradative phase. Quantitation of this and similar experiments showed that the degradation half-time of A1-reactive HA0 in the presence or absence of tunicamycin was ~6 h. It was identical to the half-time for total HA0 and total BiP in these cells. The ratio of coprecipitating labeled BiP to HA0 remained approximately constant with chase, further indicating that their association in the cell was probably permanent.

Taken together the data suggest that BiP associated rapidly and permanently with irreversibly misfolded HA0 in the ER. No transient association of BiP with any form of X:31 HA0 was detected, even at very early times after synthesis.

Discussion

The ER is the site of folding and assembly of secretory proteins and integral membrane proteins. For many proteins, including HA0, transport to the Golgi complex requires a correct tertiary and quaternary structure, implying that the cell may exert "quality control" at the level of ER to Golgi transport (Copeland et al., 1986, 1988; Lippincott-Schwartz et al., 1988; for reviews see Carlin and Merlie, 1986; Rose and Doms, 1988). For HA0, folding and trimerization is normally very efficient; up to 80% of hemagglutinin synthesized oligomerizes and is transported to the plasma membrane (Matlin and Simons, 1983; Copeland et al., 1986). However, a side pathway of folding also occurs, in which 5–10% of the HA0 produced misfolds and is retained in the ER. The misfolding of HA0 is not an artifact of virus infection since it also occurs during in vitro translation and translocation into dog pancreas microsomes (Copeland, C., unpublished results).

Inhibition of N-linked glycosylation by tunicamycin increases the amount of misfolded HA0 to 100%. A requirement for N-linked carbohydrate has been reported previously for folding of the HA0 of Influenza C (Hongo et al., 1986*a*, *b*). Mutations in the ectodomain of HA0 are also known to cause misfolding and ER retention of HA0 (Gething et al., 1986; Kozutsumi et al., 1988). ER accumulation and misfolding are also commonly, though not uniformly, observed for other mutated proteins or for glycoproteins synthesized in the presence of tunicamycin (see Rose and Doms, 1988; Schlesinger and Schlesinger, 1987).

Conformation of the Misfolded Protein

The spontaneously generated misfolded HA0 and the non-glycosylated form of HA0 produced in tunicamycin-treated cells had similar properties. Both were present as aggregates in the ER, they remained trypsin-sensitive, and they carried the same antigenic epitopes. The fact that many of these epitopes were shared with correctly folded hemagglutinin suggested that the molecules were not grossly misfolded. At least the top domains, which are made up entirely of the HA1 subunits in the mature protein, appeared normal. The stem domains, which contain the HA2 chains and two sequences from the HA1 chain, were, however, abnormal judging by their reactivity with antibody A1.

The presence of interchain disulfides was another clear indication of misfolding. In correctly folded hemagglutinin, all 12 of the ectodomain cysteines participate in intrachain disulfides (Wilson et al., 1981). The interchain disulfides must have resulted from the failure of cysteine pairing in the misfolded polypeptide chain. Apparently two or more free sulfhydryl groups remained exposed such that they could rapidly and efficiently form the aberrant interchain disul-

fides. Disulfide-bonded aggregates have been previously observed for nonglycosylated HA0 of Influenza C (Hongo et al., 1986*a*,*b*) and some mutant forms of VSV G protein (Machamer and Rose, 1988).

In HA0 only two of the six disulfides are located in the stem region where the major folding defect was likely to be located; cysteines 144 and 148 form a small, four residue loop in the HA2 chain, and cysteines 14 (of HA1) and 137 (of HA2) join the NH_2 terminus of the HA0 ectodomain to a segment close to the COOH terminus. The former disulfide does not appear structurally significant. However, the latter is responsible for connecting HA1 and HA2 subunits in the mature molecule, and is likely to be among the last of the disulfides to form. It is striking that four of the seven glycosylation sites in HA0 are close to this disulfide bond. This might explain the increased tendency for misfolding in tunicamycin-treated cells. We are presently testing whether this intrachain disulfide is missing in the misfolded HA0. The assignment of the interchain cross-links to ectodomain cysteines is further supported by our observation that cross-linked aggregates are formed when the soluble anchor-free form of HA0 misfolds (Singh, I., and A. Helenius, unpublished data).

Formation and Fate of the ER Complex

The rapidity by which the misfolded HA0 is segregated from the correctly folded HA0 monomers was remarkable. The newly synthesized polypeptides were integrated into disulfide–linked complexes immediately after chain completion. Stable BiP association and expression of the misfolded A1 epitope followed within ~3–4 min. In contrast, the correctly folded molecules require 7–10 min to trimerize. Two alternative mechanisms can be envisaged to explain these findings. The misfolded polypeptides may immediately separate from the correctly folded chains into a complex destined to be retained and eventually degraded. Alternatively, all newly synthesized HA0 molecules—correctly folded and misfolded—could enter a common, as yet unidentified, complex. The correctly folded monomers could emerge from such complexes as free subunits and proceed to trimerize, whereas misfolded molecules would not. They would remain associated with BiP in the ER. It is interesting to note that misfolded, anchor-free HA0 complexes require ~15 min to form (Singh, I., and A. Helenius, unpublished observations). This suggests that segregation of misfolded membrane proteins may be faster than soluble proteins.

The misfolded HA0 and its complexes with BiP were relatively long lived. Their half-life, 6 h, was similar to that of correctly folded HA0 trimers and BiP in these cells. The long half-time probably explains why the constitutively misfolded HA0, although a small fraction of total HA0 synthesized, appears to be so abundant in the ER (Bächi et al., 1985; Copeland et al., 1986). Whether the degradation occurs by ER-specific proteases remains to be seen. Recent studies on the fate of unassembled T-cell receptor subunits suggest that there is a separate degradative system for misfolded and unassembled proteins in the ER (Lippincott-Schwartz et al., 1988). It is possible that the presence of a lag phase followed by rapid degradation may be a common characteristic of degradation of retained proteins in the ER.

The ER Localization of Misfolded HA0

The retention of misfolded HA0 in the ER could depend upon the physical properties of the aggregated HA0 or on association with specific receptors. Aggregate size alone was, however, not likely to cause retention. The sedimentation analysis indicated an average aggregate size of ~500-kD. Some of the retained complexes were, in fact, similar in molecular mass to normal HA0 trimers (252-kD, 9 S). The exact composition of the aggregates remains unclear, but the data suggested that there are not more than one or two BiP molecules per complex.

Receptors have been postulated for the retention of BiP and other ER proteins with COOH-terminal KDEL sequences (Munro and Pelham, 1987). Recent studies by Pelham (1988*a*) suggest that such receptors function by recycling these proteins from the Golgi region to the ER. Using sensitive immunolocalization techniques, we saw no evidence for misfolded HA0 in the cisternae of the Golgi complex. The distribution was identical to that of BiP (Bole, D. G., and J. D. Jamieson, manuscript in preparation). Exclusive ER localization was also indicated by the lack of detectable trimming and endoglycosidase H sensitivity of N-linked carbohydrate side chains (Copeland et al., 1986). Our data, therefore, are not consistent with cycling of misfolded HA0 molecules between the ER and the Golgi complex. A possibility which we are pursuing is the presence of a receptor within the ER which may interact with misfolded proteins associated with BiP.

The Role of BiP

BiP is known to associate with a number of different incompletely folded, misfolded, and unassembled proteins in the ER (Bole et al., 1986; Gething et al., 1986; Dorner et al., 1987; Kassenbrock et al., 1988; Kozutsumi et al., 1988). To date, the only BiP-binding domain defined is the CH1 domain of IgG heavy chain (Hendershot et al., 1987). In the case of the misfolded HA0, the binding domain is likely to be located within the stem. The structural requirements for BiP binding remain unclear, but hydrophobic sites have been invoked (Pelham, 1986). Polypeptide flexibility and accessibility may also be important factors.

BiP association with the misfolded HA0 was noncovalent and ATP sensitive, and thus similar to what is known for other BiP complexes (Munro and Pelham, 1986; Kassenbrock et al., 1988). Even though the association appeared to be permanent within the cell, it was rather weak once the cells had been solubilized. Even with precautions, it was difficult to recover more than 50% of the misfolded HA0 in immunoprecipitates using anti-BiP. We did not detect any transient interaction between BiP and correctly folded HA0 monomers within the first minutes after synthesis. These results appear to differ from those who reported a transient interaction between monomeric HA0 of the Japan strain of influenza and BiP (Gething et al., 1986). They documented a slight reduction in the amount of BiP coprecipitating with HA0 after 15 min of pulse labeling vs. 2 h of chase. Whether this discrepancy results from differences in the virus strains or in the reactivities of the antibodies is unclear. We have recently observed a transient association of BiP with VSV G protein during the first 2 min of chase (Machamer, C. E., R. W. Doms, D. G. Bole, J. K. Rose, and A. Helenius, manu-

Script submitted for publication). Whether this indicates a role for BiP in the productive folding pathway of this protein or reflects the presence of fortuitous BiP binding sites at stages of its folding pathway remains to be elucidated.

A number of functions have been proposed for BiP. It has been suggested to serve as a folding factor (Munro and Pelham, 1986; Gething et al., 1986; Pelham, 1988*b*), as an assembly factor for oligomeric proteins (Bole et al., 1986), or as a retention factor for misfolded and unassembled polypeptides in the ER (Bole et al., 1986; Dorner et al., 1987, 1988; Kassenbrock et al., 1988). It is also possible that it might serve as a marker for proteins to be degraded in the ER. Our results do not allow exclusion of any of these potential functions for BiP's association with HA0. The lack of detectable association between BiP and monomeric HA0 during folding and trimerization, argues, however, against a crucial role of BiP in normal folding and trimer assembly. Instead, our detection of relatively long-lived complexes between BiP and nontransported forms of misfolded HA0 are most consistent with a role for BiP in ER retention. It is likely that BiP is part of the "quality control" mechanism that ensures intracellular transport is restricted to fully assembled, correctly folded proteins.

We thank the members of the Helenius, Mellman, and Jamieson laboratories for advice and criticism. We thank Drs. Skehel and Webster for anti-HA antibodies and Ann Curley-Whitehouse for photographic services.

This work was supported by grants CA 46128-01 and GM 38346-07 from the National Institutes of Health to A. Helenius. C. S. Copeland was supported by a Connecticut High Technology Graduate Fellowship and S. M. Hurtley by a European Molecular Biology Organization long-term fellowship.

Received for publication 22 November 1988 and in revised form 16 February 1989.

References

Bächi, T., W. Gerhard, and J. Yewdell. 1985. Monoclonal antibodies detect different forms of influenza virus hemagglutinin during viral penetration and biosynthesis. *J. Virol.* 55:307–313.

Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558–1566.

Boulay, F., R. W. Doms, R. Webster, and A. Helenius. 1988. Posttranslational oligomerization and cooperative acid activation of mixed influenza hemagglutinin trimers. *J. Cell Biol.* 106:629–639.

Brown, W. J., and M. G. Farquhar. 1984. The mannose-6-phosphate receptor for lysosomal enzymes is concentrated in *cis* Golgi cisternae. *Cell.* 36:295–307.

Carlin, B. E., and J. P. Merlie. 1986. Assembly of multisubunit membrane proteins. *In* Protein Compartmentalization. A. W. Strauss and I. Boime, editors. Springer-Verlag, New York. 71–86.

Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179–1191.

Copeland, C. S., K.-P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius. 1988. Folding, trimerization and transport are sequential events in the biogenesis of influenza virus hemagglutinin. *Cell.* 53:197–209.

Daniels, R. S., A. R. Douglas, J. J. Skehel, D. C. Wiley, C. W. Naeve, R. G. Webster, G. N. Rogers, and J. C. Paulson. 1984. Antigenic analysis of influenza virus haemagglutinins with different receptor-binding specificities. *Virology.* 138:174–177.

Daniels, R. S., S. Jeffries, P. Yates, G. C. Schild, G. N. Rogers, J. C. Paulson, S. A. Wharton, A. R. Douglas, J. J. Skehel, and D. C. Wiley. 1987. The receptor-binding and membrane-fusion properties of influenza variants selected using anti-haemagglutinin monoclonal antibodies. *EMBO (Eur. Mol. Biol. Organ.). J.* 6:1489–1465.

Doms, R. W., and A. Helenius. 1986. The quaternary structure of the influenza virus hemagglutinin after acid treatment. *J. Virol.* 60:833–839.

Doms, R., A. Helenius, and J. White. 1985. Membrane fusion activity of the influenza virus hemagglutinin. *J. Biol. Chem.* 260:2873–2981.

Dorner, A. J., D. G. Bole, and R. J. Kaufman. 1987. The relationship of N-linked glycosylation and heavy chain–binding protein association with the secretion of glycoproteins. *J. Cell Biol.* 105:2665–2674.

Dorner, A., M. Krane, and R. J. Kaufman. 1988. Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. *Mol. Cell. Biol.* 8:4063–4070.

Doyle, C., J. Sambrook, and M.-J. Gething. 1986. Analysis of progressive deletions of the transmembrane and cytoplasmic domains of influenza hemagglutinin. *J. Cell Biol.* 103:1193–1204.

Elbein, A. 1983. Inhibitors of glycoprotein synthesis. *Methods Enzymol.* 98:135–154.

Gething, M-J., K. McCannon, and J. Sambrook. 1986. Expression of wildtype and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* 46:939–950.

Hendershot, L., D. G. Bole, G. Kohler, and J. F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain binding protein. *J. Cell Biol.* 104:761–767.

Hongo, S., K. Sugawara, M. Homma, and K. Nakamura. 1986*a*. The functions of oligosaccharide chains associated with influenza C viral glycoproteins. 1. The formation of influenza C virus particles in the absence of glycosylation. *Arch. Virol.* 89:171–187.

Hongo, S., K. Sugawara, M. Homma, and K. Nakamura. 1986*b*. The functions of oligosaccharide chains associated with influenza C viral glycoproteins. II. The role of carbohydrates in the antigenic properties of influenza C viral glycoproteins. *Arch. Virol.* 89:189–201.

Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly. 1988. Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. *Nature (Lond.).* 333:90–93.

Kielian, M. C., M. Marsh, and A. Helenius. 1986. Kinetics of endosome acidification detected by mutant and wild-type Semliki Forest Virus. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3103–3109.

Kozutsumi, Y., M. Segal, K. Normington, M-J. Gething, and J. Sambrook. 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature (Lond.).* 332:462–464.

Lippincott-Schwartz, J., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. *Cell.* 54:209–220.

Machamer, C. E., and J. K. Rose. 1988. Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. *J. Biol. Chem.* 263:5955–5960.

Matlin, K., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell.* 34:233–243.

Munro, S., and H. R. B. Pelham. 1986. An Hsp 70-like protein in the ER: identity with the 78 kD glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell.* 46:291–300.

Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.

Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell.* 46:959–961.

Pelham, H. R. B. 1988*a*. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913–918.

Pelham, H. 1988*b*. Heat shock proteins: coming in from the cold. *Nature (Lond.).* 332:776–777.

Rose, J. K., and R. W. Doms. 1989. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* In press.

Schlesinger, M. J., and S. Schlesinger. 1987. Domains of virus glycoproteins. *Adv. Virus Res.* 33:1–44.

Verhoeyen, M., R. Fang, W. Min Jou, R. Devos, D. Huylebroeck, E. Saman, and W. Fiers. 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. *Nature (Lond.).* 286:771–776.

Webster, R. G., and W. G. Laver. 1980. Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with the selection of variants with potential epidemiological significance. *Virology.* 104:139–148.

Webster, R. G., L. E. Brown, and D. C. Jackson. 1983. Changes in the antigenicity of the hemagglutin molecule of H3 influenza virus at acidic pH. *Virology.* 126:587–599.

Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56:365–395.

Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature (Lond.).* 289:366–373.

Yewdell, J. W., A. Yellen, and T. Bächi. 1988. Monoclonal antibodies localize events in the folding, assembly and intracellular transport of the influenza virus hemagglutinin glycoprotein. *Cell.* 52:843–852.